



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 15/12, A61K 48/00, C12N 5/10,</b> <b>A61K 35/30, A61P 25/28</b>	<b>A2</b>	<b>(11) International Publication Number:</b> <b>WO 00/23587</b> <b>(43) International Publication Date:</b> 27 April 2000 (27.04.00)
<b>(21) International Application Number:</b> PCT/NL99/00638 <b>(22) International Filing Date:</b> 15 October 1999 (15.10.99)  <b>(30) Priority Data:</b> 98203480.3                      16 October 1998 (16.10.98)                      EP  <b>(71) Applicant (for all designated States except US):</b> INTROGENE B.V. [NL/NL]; Wassenaarseweg 72, NL-2333 AL Leiden (NL).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> VERLINDEN, Stefan, Frederik, Franciscus [NL/NL]; Janvossensteeg 33, NL-2312 WB Leiden (NL). VAN BEKKUM, Dirk, Willem [NL/NL]; Emmalaan 4, NL-3051 JG Rotterdam (NL).  <b>(74) Agent:</b> OTTEVANGERS, S., U.; Verenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> GENE THERAPY OF ALZHEIMER'S DISEASE BY DELIVERY OF AN ENCODED APOLIPROTEIN E		
<b>(57) Abstract</b> <p>The invention relates to Alzheimer's disease and more specifically to gene therapy and prevention of Alzheimer's disease. The invention provides a gene delivery or gene therapy vehicle and methods for its use in therapy and prevention of dementia's such as Alzheimer's disease whereby a functional ApoE gene or a functional equivalent thereof is introduced or delivered into human or humanised cells such as progenitor cells for example monocytes or glialcells.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon	KR	Republic of Korea	PL	Poland		
CN	China	KZ	Kazakstan	PT	Portugal		
CU	Cuba	LC	Saint Lucia	RO	Romania		
CZ	Czech Republic	LI	Liechtenstein	RU	Russian Federation		
DE	Germany	LK	Sri Lanka	SD	Sudan		
DK	Denmark	LR	Liberia	SE	Sweden		
EE	Estonia			SG	Singapore		

Title: GENE THERAPY OF ALZHEIMER'S DISEASE BY DELIVERY OF AN ENCODED APOLIPROTEIN E

The invention relates to Alzheimer's disease and more in specifically to gene therapy and prevention of Alzheimer's disease.

Alzheimer disease (AD) is the leading cause of dementia  
5 in the elderly, affecting approximately 5% of the population  
over age 65 which figure rises to >40% for the population  
over 85 years old. Symptoms of AD and dementia in general are  
progressive cognitive decline, in particular loss of memory,  
learning and attention. Upon pathological examination  
10 accumulation of amyloid  $\beta$  ( $A\beta$ ) in the brain is found. Other  
forms of dementia which have the development of amyloid  
plaques in common are cerebral amyloid angiopathy (CAA) and  
vascular dementia or AD with cerebrovascular disease.

The neuropathology of AD is characterised by extensive  
15 neuronal cell loss and deposition of numerous senile plaques  
and neurofibrillary tangles in the cerebral cortex. The  
major component of the senile plaques is  $A\beta$ , a 39 to 43 amino  
acid peptide derived by proteolytic cleavage from the amyloid  
 $\beta$  precursor protein (APP). Soluble  $A\beta$  species of different  
20 lengths are physiologically present in various body fluids  
including cerebrospinal fluid. The  $A\beta$  deposits found in the  
brain occur as extra-cellular diffuse plaques, as well as  
neuritic plaques containing dense cores surrounded by  
dystrophic neurites. Neurofibrillary tangles are found  
25 intraneuronally and are composed mainly of paired helical  
filaments containing a hyperphosphorylated form of the  
microtubule-associated protein tau. Regions that are  
affected most are the temporal lobes and the frontal,  
parietal and posterior cingulate cortices which areas are  
30 associated with cognitive functions.

The underlying disease mechanism is not yet understood although some genetic linkage is found which led to the finding of two distinct forms, early or late onset AD.

1) Early onset or familial AD. Five percent of all AD patients develop disease before age 60. In these patients mutations of the APP, the presenilin1 gene or the presenilin2 gene located on chromosomes 21, 14 or 1 are found. These mutations have in common that they alter APP or A $\beta$  processing which results in plaque formation and AD.

2) Late onset or sporadic AD, the vast majority of all patients, with a disease onset after age 60. In these patients a linkage to a region on chromosome 19 was shown, where the apolipoprotein E (APOE for the gene and ApoE for the protein) locus is found (Pericak-Vance and al. 1991).

ApoE is a 34 kilodalton plasma protein, that binds to the low-density lipoprotein receptor and/or the LDL receptor-related protein a/2-macroglobulin receptor (LRP) and is involved in the transport of cholesterol and other lipids in various cells in the body. The LRP receptor is present on brain neurons. Mutational analysis showed that region 136-160 of ApoE is critical for LDL receptor interaction.

The majority of the plasma ApoE is derived from the liver where it is synthesised by hepatocytes. In other organs and tissues it is also synthesised, albeit in much lower levels, by monocyte derived macrophages. In the different organs/tissues macrophages have different names for instance microglial cells in the brain. In the brain, after the liver the organ which produces the second most ApoE, the protein is synthesised by glial cells from which originally only microglial cells were thought to be monocyte derived. Recently it was shown that macroglial cells are also monocyte derived (Eglitis and Mezey, 1997) so all glial cells are monocyte derived.

ApoE is found in humans by isoelectric focusing in three major isoforms (E2, E3 and E4), minor isoforms in humans are E1, E5, E6 and E7. Within these isoforms different mutations can be found which result in yet further differing proteins having the same isoelectric focusing point (Weisgraber 1994). Some examples of isoform mutations are ApoE2-christchurch, ApoE2-Heidelberg, ApoE1-Harrisburg, ApoE4-Philadelphia and ApoE3-Leiden (de Villiers, 1997).

The three major allelic variants are found in a frequency of 77% for e3, 15% for e4 and 7% for e2 and they differ by single amino acid substitutions at positions 112 and 158: E3 (Cys 112, Arg 158), E4 (Cys112Arg) and E2 (Arg158Cys).

Further studies in late onset AD revealed that the frequency of APOE e4 is increased in AD patients and that there is a gene dose effect (Strittmater and al. 1993). Not only the risk for AD is increased significantly also the mean age of onset of AD decreases with an increasing number of APOE e4 alleles from >75 for 0 alleles to <70 when two alleles are present (Higgins, 1997). It also was found that subjects carrying one or two APOE e2 alleles are protected from the disease and if they get AD the mean age of onset is >80 years of age. When the chance of getting AD in the age group 60-65 is set to 1 for subjects with the phenotype e3/e3, the chance is 0.1 for subjects with the phenotype e2/e3, 1.1 with e2/e4, 11.1 with e3/e4 and 123.8 with e4/e4 (Corder, 1994).

It is suggested that ApoE amongst others is involved in the clearing of A $\beta$  (Aleshkov, 1997). The idea is that AD is actually a processing disorder. This seems to be supported by the finding that AD occurs in Down syndrome patients without mutations in APP, presenilin1 or presenilin 2 with a disease onset before age 60. Due to trisomy of chromosome 21, which contains the APP gene, Down syndrome patients express 1.5 times the amount of APP as compared to normal humans.

Typically these patients start developing AD from age 40. In different studies both an increased risk for AD when one or two APOE $\epsilon$ 4 alleles are present (Schupf, 1998) as a protective effect of one or two APOE $\epsilon$ 2 alleles are found (Tyrrel, 1998).

5 In conclusion enhancement of APP, the A $\beta$  precursor protein, leads to earlier onset of AD with a similar distribution of APOE phenotypes in Down syndrome patients and normal persons.

Human primates are the only species known to possess at least three different APOE isotypes. All other known species, 10 except rabbits who carry APOE  $\epsilon$ 3, only carry the APOE  $\epsilon$ 4 isotype (Poduri, 1992). From the group of non-human primates only Rhesus monkeys were described to develop AD like pathology at age >25 years. This is a major obstruction in the study for late onset AD because no easy accessible animal 15 model is available and a study in Rhesus monkeys would cost several decades, depending on the strategy. The study of early AD is possible in rodents and other small animals, since the genes with the same mutations found in men (i.e. APP, presenilin2 or presenilin2) when introduced in 20 transgenic mice gives similar pathology. However, the observed APOE-gene in man is not found in mice, so it seems that the mode of action of ApoE in mice differs from that found in man. Recently it was found that in APOE -/-knock out mice age related congophilic inclusions in the brain occur 25 which are to a much lesser extent present in APOE +/- parental mice (Robertson, 1998). These congophilic inclusions consist of ubiquitin and A $\beta$  and are found predominantly in protoplasmic astrocytes or macroglial cells in the dorsal hippocampus, a location where one expects to find plaques in 30 AD patients. Other areas are the piriform cortex and cerebellum.

The present invention relates to the field of human gene therapy, more in particular to novel gene therapy vehicles to

alter the ApoE phenotype of human cells. As described above, the ApoE phenotype of the glial cells in the brain is related to the risk of development of dementia such as Alzheimer disease. For the purpose of allowing gene therapy aimed at the reduction of this risk, the invention provides gene therapy vehicles suitable to alter the ApoE phenotype of human cells.

The invention provides a gene delivery or gene therapy vehicle and methods for its use in therapy and prevention of dementia's such as Alzheimer's disease whereby a functional APOE gene or a functional equivalent thereof is introduced or delivered into human or humanised cells such as progenitor cells for example monocytes or glial cells. Monocytes and subsequently glial cells originate from hematopoietic stem cells in the bone marrow. It has been shown that bone marrow transplantation's may lead to complete and long-lasting chimerism of hematopoietic stem cells and their progeny. In the host, monocyte derived tissue macrophages (i.e. glial cells) will be replaced by donor macrophages. This technique is used for instance to replace glial cells in patients with lysosomal storage diseases based on a enzyme defect, by bone marrow transplantation with macrophages from a healthy donor which contain the correct genetic information of the affected enzyme (Hoogerbrugge, 1988). Similar experiments have been performed in artherosclerotic mice. Since the main systemic function of ApoE is clearance of lipoproteins from the plasma, APOE -/- mice also develop hyperlipoproteinemia. High lipoprotein levels cause precipitation of these proteins in these artery vessel wall leading to atheroscleroses probably due to inadequate processing by local tissue macrophages. Transplanting bone marrow from APOE +/+ mice into APOE -/- mouse is sufficient to reduce the formation of arterioscleroses, although the systemic levels of lipoproteins are not corrected (Linton, 1995). The other way

around 1f APOE +/+ mice are transplanted with -/- bone marrow, these chimeric animals became more prone to develop arterioscleroses although their serum level of lipoproteins in normal animals because their liver produces sufficient amounts of ApoE. So in conclusion local production of ApoE by tissue macrophages is sufficient to prevent storage of lipoproteins leading to decreased arterioscleroses formation.

The invention provides a gene delivery vehicle comprising a APOE or APOE -like gene construct or functional fragment thereof that is expressed in the host cell to alter or modify the APOE phenotype of the host cell, more specifically to provide an APOE phenotype which results in an elevation of the amyloid  $\beta$  processing activity of the transfected cell. In a preferred embodiment, a gene delivery vehicle according to the invention is provided with a gene or fragment thereof comprising the e2 or e3 apolipoprotein E gene or functional fragment thereof. These genes or fragments encode gene products (proteins) which have superior amyloid  $\beta$  processing capacity over those of for example e4.

In a preferred embodiment the invention provides a gene delivery vehicle further comprising a secretion signal allowing secretion of a gene product of said gene or fragment thereof. Most of the APOE genes of the invention comprise a secretion signal for transport of the protein to the exterior of the cell, however, additional secretion signals may be added to enhance specific secretion in glial cell progenitors and their progeny.

The invention herewith provides a method for providing a cell with a higher amyloid  $\beta$  processing capacity than it had before comprising treating said cell with a gene delivery vehicle according the invention. The cells, preferably glial-cell progenitor cells (monocytes or even stem cells) with the altered phenotype find application in human gene therapy



aimed at the reduction of the risk, onset or development of dementia's such as Alzheimer Disease.

In this manner cells such as glial cells are provided which may originate from individuals which APOE phenotype  
5 enhances the development of AD but are now modified or changed to an ApoE phenotype which decreases the risk on development of AD.

The invention herewith provides a method for providing an individual with a higher amyloid  $\beta$  processing capacity  
10 than said individual had before comprising providing said individual with at least one cell according to the invention. There are several methods to perform this phenotype switch, two possible examples are:

Modification of the committed progenitors of glial cells  
15 by a gene delivery vehicle according to the invention such as an adenoviral, AAV or other viral or non-viral vector as provided by the invention. Monocytes, the progenitors of tissue macrophages/glial cells are collected from patients by leucophoresis. Upon infection with a gene delivery vehicle  
20 according to the invention containing the desired APOE gene or functional fragment thereof, cells are transplanted back into the patient where the cells will migrate into their "end tissues" and differentiate into tissue macrophages/glial cells. Since the cells do not divide expression of the  
25 transgene occurs during the lifespan of the cell which is 0.5-2 years. This method has to be repeated every year to assure that sufficient numbers of transduced glial cells remain present (>0.5% of all glial cells).

Transduction of pluripotent hematopoietic stem cells  
30 (HSC) by a gene delivery vehicle according to the invention, such as an integrating retroviral vector containing the desired APOE transgene.

HSC are obtained by bone marrow puncture or from the peripheral blood by leucopheresis from "mobilised" patients,

for instance with GM-CSF. Upon purification of the fraction containing the HSC these cells are transduced, for instance by co-cultivation with the virus producer cells. Patients are pre-treated, if necessary with a myeloablative therapy, radiation, chemotherapy or a combination of both (Havenga, 1997). In a preferred embodiment, the invention provides a method of treatment wherein said individual is having an known increased risk of development of dementia such as Alzheimer disease, but patients with unknown risks can also be treated, for example with a cell comprising a gene delivery vehicle comprising an e2 gene or functional fragment thereof.

A preferred retroviral vector as described in WO93/07281 containing chimeric LTR's of MoMLV and MoMSV and a mutant polyoma enhancer PyF101. Furthermore the retroviral particle can contain mutant or chimeric envelope proteins that enter the HSC through the human homologue of the murine ecotropic virus receptor. Alternatively a mutant retroviral envelope is used that is optimised for HSC using envelope display libraries.

With the development of gene therapy it becomes feasible to change the phenotype of mammalian cells by transducing cells with a vector containing a gene of interest. Upon infection and depending on the vector the genetic information will be either stable integrated into the hosts genome or the vector will remain present as episomal DNA. In both cases, depending on the promoter and/or the locus control region present in the vector, gene transcription can take place.

The invention provides a method to alter the development of dementia by changing the APOE phenotype of glial cells in the brain from one that gives an increased risk to develop AD to one that gives a normalised or reduced risk to develop AD. This is based on the finding that the presence of apolipoprotein E (gene) containing monocyte derived microglial and macro glial cells in the brain are sufficient

to delay the formation of congophilic inclusions in Apolipoprotein knockout mice.

A gene therapy vehicle according to the invention may for example comprise an adenovirus, an adeno associated virus, or a retrovirus derived vector or a non-viral vector  
5 for delivery of the APOE gene construct.

The invention is further exemplified in the experimental part of the description which is not limiting the invention.

## 10 **Materials and methods**

### ApoE2 retroviral vector

Both ecotropic and amphotropic retroviral vectors containing  
15 the APO E2 cDNA are generated as previously described (Vogels, 1996).

Briefly, the APOE e2 cDNA is cloned into pLEC using a unique restriction site present in the polylinker of pLEC thus generating IG-APOEe2-1. The sequence of APOE e2 is derived  
20 from the sequence given in Genbank (locus HUMAPOE3), by changing the nucleotide C into T at position number 586, as is said in the text given with the sequence. The APOE e2 cDNA is ligated to BamHI-linkers (#1065, Biolabs, Beverly, MA), digested with BamHI and ligated into BamHI digested pLEC  
25 resulting in the vector IG-APOEe2-1. Subsequently, IG-APOEe2-1 is digested with NheI which is present in both the 5'- and 3'-LTR of pLEC. The NheI fragment of IG-APOEe2-1 containing the APOE e2 gene is isolated from agarose gel separation technique and purified by using a Gene clean kit (BIO-101  
30 Inc, Ca, USA). This fragment is cloned into the NheI site of construct pBR.dMo+PyF101 to generate a retroviral construct coded IG-APOEe2-2. This construct is used to generate recombinant retroviruses in the following manner:

Ten mg of pIG-APOEe2-2 and one mg of pCMV-Neo are co-transfected by calcium precipitation (Gibco, according to manufacturers protocol) into GP+E86 ecotropic producer cells. Stably transfected cells are selected by adding 1 mg/ml of G418 and culture the cells for 7 days. G418-resistant cells, as demonstrated by killing of parental GP+E86 cells, are pooled and tested for ApoE e2 expression and virus production by detection of ApoE e2 in the producer cells and in NIH/3T3 fibroblasts infected with supernatant generated by these producer cells.

Cell culture supernatant derived from G418 resistant, ApoE e2 expressing GP+E86 producer cells is used to infect the amphotropic retrovirus producer cell line PA317. This infection is performed by adding 1 ml GP+E86 derived virus supernatant to  $10^4$  PA317 cells in the presence of 4 mg/ml protamine HCL in a 24 wells plate. After 48 hrs the cells are harvested by trypsinisation. A cloned retroviral producer cell line is obtained by performing two rounds of limiting dilution in 96-well plates. For this purpose, infected PA317 cells are seeded at a concentration of 0.3 cell per well in 100  $\mu$ l Dulbeco's modified eagles medium (DMEM). Fifty independent clones are screened for expression of ApoE e2 protein in the supernatant of the individual producer clones and in the supernatant of NIH/3T3 cells infected with virus supernatant of this clone. The clone which expresses the highest amount of ApoE e2 and upon infection gives the highest titer of APOE e2 in NIH/3T3's is selected, coded PA317-APOEe2 and used for retrovirus production. Virus supernatant from this clone is also used to infect ecotropic GP+E86 virus producer cells, and the highest expression clone is selected similarly as described above. This clone is coded GP+E86-APOEe2 and is used to produce an ecotropic recombinant retrovirus batch. A preferred retroviral vector as described

in WO93707281 containing chimeric LTR's of MoMLV and MoMSV and a mutant polyoma enhancer PyF101. Furthermore the retroviral particle can contain mutant or chimeric envelope proteins that enter the HSC through the human homologue of the murine ecotropic virus receptor. Alternatively a mutant retroviral envelope is used that is optimised for HSC using envelope display libraries, for example which enter HSC cells through hCAT1.

10 Human umbilical cord blood (CB)

CB mononuclear cells (MNC) are separated by a Ficoll density gradient and used for ApoE phenotyping and purification of CD34+ hematopoietic progenitor cells.

15 CD34+ hematopoietic progenitor cells used for *in vitro* culture of monocytes are separated by magnetic activated cell sorting (MACS, Miltenyi, Germany) according to the manufacturers protocol. Primary monocytes are separated by seeding the CD34<sup>-</sup> cell fraction on tissue culture plastic in DMEM and incubate cells for one hour at 37°C. After one hour the non adherent cells are removed and the remaining cells are washed twice with phosphate buffered saline (PBS). The remaining cells are mainly monocytes as shown by CD14 positive staining in a Flow cytometric assay.

25

Transduction of CD34+ cells

10<sup>5</sup> APOE e2 negative CD34+ cells are seeded in a 24 wells plates which contains a monolayer of lethally irradiated PA317-IG-APO E2 producer cells in the presence of 10 µg/ml rhuIL-3 and rhuGM-CSF and 4 µg/ml protamine HCL.

30 In this manner cells are differentiated into the myelocytic lineage and at the same time these cells are transduced with

the retroviral vector by co-cultivating them with virus producer cells. After 7-14 days the supernatant is harvested to determine ApoE e2 expression.

5 Animals

Homozygous APOE -/- knockout mice we used either from the Jackson Laboratory (Bar Harbor, Maine, USA) Hill (Piedrahita, 1992) or Leiden University Medical Center (Leiden, The Netherlands) (van Ree, 1995). Both mice strain are derived from C57BL/6 X 129 hybrids which are backcrossed in C56BL/6J mice. As APOE +/+ mice C57BL/6 mice are used, as a result the two strains are, except for the APOE -/- mutation genetically identical.

15

Bone marrow transplantation

APOE -/- new-born mice, age 1 to 6 days, are lethally irradiated with 5-10 Gy, 3 hours before transplantation with 1.0-10x10<sup>6</sup> bone marrow cells obtained from APOE +/+ donor mice. Donor mice of 4-6 weeks old are used as donor to obtain pseudoautologous (congenic) bone marrow cells. As control groups new-born APOE -/- mice are transplanted with 1.0-10x10<sup>6</sup> BM cells from 4-6 weeks old APOE -/- mice and APOE +/+ new-born mice are transplanted with 1.0-10x10<sup>6</sup> BM cells from 4-6 weeks old APOE +/+ mice. In each group 24 mice are transplanted as described above. Starting after 6 weeks, every 4 weeks until week 26, 4 mice of each group were euthanized.

30

Transduction of mouse bone marrow (BM)

Transduction of murine BM by co-cultivation occurred as described previously (van Beusechem, 1990). Briefly BM of adult APOE -/- mice is harvested and enriched for progenitors by a metrizamide density gradient (sp.gr.<1.08 g/cm<sup>3</sup>). One million low density cells are co-cultivated for 72 hrs with a 70% confluent irradiated (20 Gy) monolayer of HCL on ecotropic IG-human ApoE2 or IG-hu NGFR virus producer cells, supplemented with recombinant human IL-1a, recombinant murine IL-3 and 0.4 µg/ml protamine-HCL. After 72 hrs cells are collected and injected into sub-lethally irradiated new-born APOE -/- mice. Groups of three mice are killed every 4 weeks starting at week 6 until week 24. Brains from these mice are removed, fixated in paraformaldehyde 4% and human APOE e2 in combination with an murine glial cell marker.

#### Histology

Mice brains are pre-fixated by perfusing the anaesthetised animals with 50 ml phosphate buffered saline followed by 50 ml of 4% paraformaldehyde in 0.05 M phosphate buffer, pH 7.4. The excised brains are cut in 2 mm slices in a coronal mouse brain matrix and re-immersed in the same fixative for 24 hrs. The slices containing sections of the hippocampal area are paraffin embedded in it's coronal plane. Eight mm paraffin sections are cut and mounted onto glass slides and from each slice always two slides are prepared which are stained with Haematoxylin or Eosin and Periodic Acid-Schiff reagent. Sections for further staining with antibodies are pre-treated with Histomouse blocking kit (Zymed, USA) according to the manufacturers protocol. All labelled and stained sections are examined with either an Olympus light microscope or a Zeiss Axiophot with Plan-NeoFLUAR objectives.

### Staining of congophilia

Paraffin embedded coronal section of 10 mm were stained with  
5 Congo red and prepared for fluorescent detection of amyloid  
depositions as described by Askansas (Askansas, 1993). Slides  
were viewed in bright field polarised light by  
epifluorescence illumination, using two filter combinations  
for fluorescence analyses: 1. fluorescein isothiocyanate  
10 (FITC) filters (475- to 495-nm exciter filter, 520 nm barrier  
filter and 510-nm dichroic filter) or 2. Texas red filters  
(530- to 585- exciter filters, 615-nm barrier filters and 600-  
nm dichroic filter).

### 15 Amyloid $\beta$ staining

Sections are stained as described by Robertson (Robertson,  
Dutton et al. 1998). Briefly, after microwave retrieval, a  
wash step with Tris-buffered saline (TBS) and blocking with  
20 Histomouse blocking kit the slides are stained with a  
specific monoclonal antibody against the 17-24 amino acid  
sequence of human A $\beta$ . This antibody 4G8 (Senetek, Maryland  
heights, MO, USA) cross-reacts with murine A $\beta$ .

### 25 Human and murine apolipoprotein E staining in tissue sections

To show that donor bone marrow derived glial cells found in  
the brains of APOE -/- mice are derived from the transplanted  
hematopoietic progenitors of either the syngeneic transduced  
30 or APOE +/+ donor hematopoietic grafts, 5 serial paraffin  
embedded sections are cut and stained with a mouse monoclonal  
antibody to human APOE e2 (F48.1, Research Diagnostics Inc,  
NJ, USA) or rabbit polyclonal antibody to mouse ApoE  
(Biodesign International, Kennebunkport, ME).



Human APOE e2 in supernatant

ApoE e2 expression levels in cell suspension and/or cellular  
5 supernatant are determined by Western blotting.

For cell-suspensions all adherent cells derived from each CB  
sample are collected after washing twice with PBS by scraping  
and lysing in RIPA (1%NP-40, 0.5% sodium deoxycholate and  
0.1% SDS in PBS supplemented with 1 mM

10 phenylmethanesulfonylfluoride and 0.1 mg/ml trypsin  
inhibitor). After 15 minutes incubation on ice, the lysates  
are cleared by centrifugation. Protein concentrations are  
determined by the Bio-Rad protein assay, according to the  
manufacturers protocol (BioRad). Equal amounts of whole cell  
15 extract are fractionated by SDS-PAGE on 10% gels. Proteins  
are transferred onto Immobilon-P membranes (Millipore) and  
incubated with the ApoE e2 monoclonal antibody F48.1  
(Research Diagnostics Inc, NJ, USA). The secondary labelling  
is done with a horseradish-peroxidase conjugated goat anti-  
20 mouse antibody (BioRad). Both procedures are done according  
to the protocol provided by Millipore. Antibody complexes are  
visualised with the ECL detection system according to the  
manufacturers protocol (Amersham)

Human ApoE e2 expression in permeabilised cells

Primary monocytes from individual samples are upon removal by  
scraping fixated and permeabilised as follows:  $5 \times 10^5$  cells  
are washed in ice-cold PBS. Pelleted cells are re-suspended  
30 in 0.5 ml ice-cold PBS to which 0.5 ml ice-cold 2% phosphate  
buffered paraformaldehyde (ph 7.2) containing 160 mg/ml L-a-  
lysophosphatidylcholine is added drop-wise under constant  
swirling. The cell suspension is kept for 5 min on ice

whereafter the reaction is stopped by adding 2 ml PBS containing 0.5% bovine serum albumin (BSA). Cells are pelleted and re-suspended in 100 µl PBS/0.5%BSA, stained with 10 µl (50 µg/ml) antibody to human ApoE e2 (Clone F48.1, Research Diagnostics Inc, NJ, USA) and stored for 30 minute at +4°C. After washing and re-suspending the pellet in 100 µl PBS/0.5%BSA, 10 µl (50 µg/ml) rat anti mouse conjugated with phycoerythrin (PE) (Becton and Dickinson (B+D), CA, USA) is added and the sample stored for 30 min at +4°C. After one additional wash step the cell pellet is re-suspended in 500 µl PBS/0.5%BSA and analysed on a FACSsort (B+D) according to the manufacturers protocol. Data is analysed with the CELLquest acquisition and analyses program (B+D).

#### 15 Murine macrophages and glial cells

Paraffin embedded coupes of the brain are stained with either a rat monoclonal antibody to mouse macrophage, NOMA-2 (Accurate chemicals) or a polyclonal mouse or rabbit antibody against the astroglial marker GFAP (Zymed, USA) that cross reacts with murine GFAP to detect macrophages and glial cells in the brain.

#### Secondary antibody staining

Sections stained with single and double unlabelled primary antibodies are washed and incubated with the appropriate APAAP or PAP single-stain or APAAP/PAP doublestain visualisation kit (DAKO, Glostrup, Denmark). After this procedure all slides are counter stained with hematoxylin.

**Results:**CB MNC

- 5 FACS analyses of more than 50 individual CB samples revealed that there is a clear difference in expression between ApoE e2 negative monocytes in and ApoE e2 positive monocytes (table 1).
- 10 Upon transduction of CD34+ progenitors derived monocytes from a APOE E2- donor is found that expression levels are comparable with the wild type level (table 1).

CD14+ monocytes	untransduced (peak signal)	transduced (peak signal)
fresh		
ApoE e2 -	5±2	nd
ApoE e2 +	1000±250	nd
cultured		
APOE E2 -	7±2	1050±260
APOE E2 - (mock infected)	9±3	10±3

15 *Table 1: ApoE e2 expression in of fresh and cultured transduced human monocytes.*

Effect of bone marrow transplantation on occurrence of amyloid b containing inclusions

- 20 Upon examination of the paraffin section of the brains of animals from the three groups by light microscopy, clusters of PAS positive granular structures were regularly found in

the brains of the control mice and of the parental strain mice transplanted with APOE -/- BM. Clusters were found predominantly in the hippocampal region. In the APOE-/- mice transplanted with parental strain BM occasional clusters of granules were found but to a much lower extent than in the control groups (table 2).

group	6 weeks	10 weeks	14 weeks	18 weeks	22 weeks	26 weeks
APOE +/+ → APOE +/+	0.1	0.1	0.3	0.5	0.8	1.3
APOE +/+ → APOE -/-	0.1	0.3	0.9	1.5	2.5	5
APOE -/- → APOE -/-	1	5	10	18	26	43
APOE -/- → APOE +/+	0.8	1.5	4	8	15	20

Table 2: number of PAS positive granular structures in the Hippocampal region.

Chimerism and murine and human ApoE expression in the brain

Analyses of the brainsections from APOE -/- mice transplanted with APOE +/+ BM revealed that ApoE positive monocytes/glial cells are present in the brains of APOE -/- mice transplanted with APOE +/+ BM (table 3).

These double positive cells weren't seen in APOE -/- mice transplanted with BM from APOE -/- mice. No ApoE negative

monocytes/glial cells found in APOE +/+ mice transplanted with APOE +/+ BM.

week	% donor derived cells
4	4
8	9
12	15
16	22
20	30
24	36

- 5 *Table 3: percentage of donor derived glial cells in APOE -/- mice transplanted with APOE +/+ bone marrow.*

- 10 Analyses of brainsections of APO -/- mice transplanted with GP+E86-IG-APOEe2 transduced APOE -/- BM showed similar results as above showing that the human APOEe2 transgene is expressed in BM derived brain glial cells (table 4).

week	% APOE e2+ donor cells
4	2
8	5
12	11
16	14
20	20
24	26

- 15 *Table 4: percentage of ApoE e2 positive donor cells in APOE -/- mice transplanted with GP+E86-IG-ApoE2 transduced APOE -/- bone marrow.*

20

**REFERENCES**

Aleshkov, S. and e. al. (1997). "Interaction of nascent ApoE2, ApoE3 and ApoE4 isoforms expressed in mammalian cells with amyloid peptide b (1-40). Relevance to Alzheimer's disease." Biochemistry **36**: 10571-10580.

5

Askansas, V., W. K. Engel, et al. (1993). "Enhanced detection of Congo-red-positive amyloid depositions in muscle fibers of inclusions body myositis and brain of Alzheimer's disease using fluorescence technique." Neurology **43**: 1265-1267.

10

Corder, E. H. and e. al. (1994). "Protective effect of apolipoprotein E type 2 allele for late onset Alzheimer's disease." Nature Genet **7**: 180-184.

15

de Villiers, W. J. S., D. R. van der Westhuyzen, et al. (1997). "The apolipoprotein E2 (Arg145Cys) mutation causes autosomal dominant type III hyperlipoproteinemia with incomplete penetrance." Arterioscler Thromb Vasc Biol **17**: 865-872.

20

Eglitis, M. A. and E. Mezey (1997). "Hematopoietic cells differentiate into both microglia and macroglia in the brains of adult mice." PNAS **94**: 4080-4085.

25

Havenga, M. (1997). "Retroviral stem cell gene therapy." Stem Cells **15**: 162-179.

Higgins, G. A., C. H. Large, et al. (1997). "Apolipoprotein E and Alzheimer's disease: a review of recent studies."

30

Pharmacol Biochem Behav **56**(4): 675-685.

Hoogerbrugge, P. M. (1988). Bone marrow transplantation for lysosomal enzyme deficiency. Department of Pediatrics. Leiden, University Hospital Leiden.

5

Linton, M. F., J. B. Atkinson, et al. (1995). "Prevention of arteriosclerosis in apolipoprotein E-deficient mice by bone marrow transplantation." Science **267**: 1034-1037.

10 Pericak-Vance, M. A. and e. al. (1991). "Linkage studies in familial Alzheimer's disease: evidence for chromosome 19 linkage." Am J hum Genet **48**: 1034-1050.

Piedrahita, J. A., S. H. Zhang, et al. (1992). "Generation of mice carrying a mutant apolipoprotein E gene inactivated by gene targeting in embryonic stem cells." PNAS **89**: 4471-4475.

Poduri, A., M. Gearing, et al. (1992). "Apolipoprotein E4 and beta amyloid in senile plaques and cerebral blood vessels of aged Rhesus monkeys." Am J Pathol **6**: 1183-1187.

Ree, J. H. van den Broek, M. J. Hofker, m. H. Havekse, L. M. (1995). "Atypical xanthomatosis in apolipoprotein E-deficient mice after cholesterol feeding." Atherosclerosis **112** (2): 237-243

Robertson, T. A., N. S. Dutton, et al. (1998). "Age-related congophilic inclusions in the brains of apolipoprotein E-deficient mice." Neuroscience **81**(1): 171-180.

30

Schupf, N., D. Kapell, et al. (1998). "Earlier onset of Alzheimer's disease in men with Down syndrome." Neurology **50**: 991-995.

Strittmater, W. J. and e. al. (1993). "Apolipoprotein E: high-avidity binding to b-amyloid and increase frequency of type e4 allele in late-onset familial Alzheimer's disease."

5 PNAS **90**: 1977-1981.

Tyrrel, J., M. Cosgrave, et al. (1998). "A protective effect of apolipoprotein E e2 allele on dementia in Down's syndrome." Biol Psychiatry **43**: 397-400.

10

van Beusechem, V. W. Kukler, A. Einerhand, M. P. W. Bakx, T. A.

van der Eb, A. J. van Bekkum, D. W. and Valerio D. (1990)

15 "Expression of human adenosine deaminase in mice transplanted with hemopoietic stem cells infected with amphotropic retroviruses." J. Exp. Med. **172**: 329-336

Vogels, R. Boesen, J. van Es, H.H.G van Beuschem, V.W.

Valerio, D. (1996). "Improved retroviral vectors especially

20 suitable for gene therapy." patent application Wo96/35798

Weisgraber, K. H. (1994). "Apolipoprotein E: structure-function relationships." Adv Prot Chem **45**: 249-302.



**CLAIMS**

1. A gene delivery vehicle comprising an apolipoprotein E gene or functional fragment thereof.
2. A gene delivery vehicle according to claim 1 for delivery of said gene or functional fragment thereof to a human cell.
- 5 3. A gene delivery vehicle according to claim 2 wherein said cell is a glial-cell progenitor cell.
4. A gene delivery vehicle according to anyone of claims 1 to 3 wherein said gene comprises the e2 or e3 apolipoprotein E gene.
- 10 5. A gene delivery vehicle according to anyone of claims 1 to 4 further comprising a secretion signal allowing secretion of a gene product of said gene or said fragment thereof.
6. A method for providing a cell with a higher amyloid  $\beta$  processing capacity than it had before comprising treating  
15 said cell with a gene delivery vehicle according to anyone of claims 1 to 5.
7. A cell, preferably a glial-cell progenitor cell or the progeny thereof, comprising a gene delivery vehicle according to anyone of claims 1 to 5.
- 20 8. A method for providing an individual with a higher amyloid  $\beta$  processing capacity than said individual had before comprising providing said individual with at least one cell according to claim 7.
9. A method according to claim 8 wherein said individual is  
25 suffering from dementia such as Alzheimer disease or wherein said individual has an increased risk of developing said dementia.





## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 15/12, A61K 48/00, C12N 5/10,</b> <b>A61K 35/30, A61P 25/28</b>	<b>A3</b>	<b>(11) International Publication Number:</b> <b>WO 00/23587</b> <b>(43) International Publication Date:</b> 27 April 2000 (27.04.00)
<b>(21) International Application Number:</b> PCT/NL99/00638 <b>(22) International Filing Date:</b> 15 October 1999 (15.10.99) <b>(30) Priority Data:</b> 98203480.3                      16 October 1998 (16.10.98)                      EP <b>(71) Applicant (for all designated States except US):</b> INTROGENE B.V. [NL/NL]; Wassenaarseweg 72, NL-2333 AL Leiden (NL). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> VERLINDEN, Stefan, Frederik, Franciscus [NL/NL]; Janvossensteeg 33, NL-2312 WB Leiden (NL). VAN BEKKUM, Dirk, Willem [NL/NL]; Emmalaan 4, NL-3051 JG Rotterdam (NL). <b>(74) Agent:</b> OTTEVANGERS, S., U.; Vereenigde Octrooibureaux, Nieuwe Parklaan 97, NL-2587 BN The Hague (NL).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i>  <b>(88) Date of publication of the international search report:</b> 3 August 2000 (03.08.00)
<b>(54) Title:</b> GENE THERAPY OF ALZHEIMER'S DISEASE BY DELIVERY OF AN ENCODED APOLIPROTEIN E  <b>(57) Abstract</b> <p>The invention relates to Alzheimer's disease and more specifically to gene therapy and prevention of Alzheimer's disease. The invention provides a gene delivery or gene therapy vehicle and methods for its use in therapy and prevention of dementia's such as Alzheimer's disease whereby a functional ApoE gene or a functional equivalent thereof is introduced or delivered into human or humanised cells such as progenitor cells for example monocytes or glialcells.</p>		

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

<b>AL</b>	Albania	<b>ES</b>	Spain	<b>LS</b>	Lesotho	<b>SI</b>	Slovenia
<b>AM</b>	Armenia	<b>FI</b>	Finland	<b>LT</b>	Lithuania	<b>SK</b>	Slovakia
<b>AT</b>	Austria	<b>FR</b>	France	<b>LU</b>	Luxembourg	<b>SN</b>	Senegal
<b>AU</b>	Australia	<b>GA</b>	Gabon	<b>LV</b>	Latvia	<b>SZ</b>	Swaziland
<b>AZ</b>	Azerbaijan	<b>GB</b>	United Kingdom	<b>MC</b>	Monaco	<b>TD</b>	Chad
<b>BA</b>	Bosnia and Herzegovina	<b>GE</b>	Georgia	<b>MD</b>	Republic of Moldova	<b>TG</b>	Togo
<b>BB</b>	Barbados	<b>GH</b>	Ghana	<b>MG</b>	Madagascar	<b>TJ</b>	Tajikistan
<b>BE</b>	Belgium	<b>GN</b>	Guinea	<b>MK</b>	The former Yugoslav	<b>TM</b>	Turkmenistan
<b>BF</b>	Burkina Faso	<b>GR</b>	Greece		Republic of Macedonia	<b>TR</b>	Turkey
<b>BG</b>	Bulgaria	<b>HU</b>	Hungary	<b>ML</b>	Mali	<b>TT</b>	Trinidad and Tobago
<b>BJ</b>	Benin	<b>IE</b>	Ireland	<b>MN</b>	Mongolia	<b>UA</b>	Ukraine
<b>BR</b>	Brazil	<b>IL</b>	Israel	<b>MR</b>	Mauritania	<b>UG</b>	Uganda
<b>BY</b>	Belarus	<b>IS</b>	Iceland	<b>MW</b>	Malawi	<b>US</b>	United States of America
<b>CA</b>	Canada	<b>IT</b>	Italy	<b>MX</b>	Mexico	<b>UZ</b>	Uzbekistan
<b>CF</b>	Central African Republic	<b>JP</b>	Japan	<b>NE</b>	Niger	<b>VN</b>	Viet Nam
<b>CG</b>	Congo	<b>KE</b>	Kenya	<b>NL</b>	Netherlands	<b>YU</b>	Yugoslavia
<b>CH</b>	Switzerland	<b>KG</b>	Kyrgyzstan	<b>NO</b>	Norway	<b>ZW</b>	Zimbabwe
<b>CI</b>	Côte d'Ivoire	<b>KP</b>	Democratic People's	<b>NZ</b>	New Zealand		
<b>CM</b>	Cameroon		Republic of Korea	<b>PL</b>	Poland		
<b>CN</b>	China	<b>KR</b>	Republic of Korea	<b>PT</b>	Portugal		
<b>CU</b>	Cuba	<b>KZ</b>	Kazakstan	<b>RO</b>	Romania		
<b>CZ</b>	Czech Republic	<b>LC</b>	Saint Lucia	<b>RU</b>	Russian Federation		
<b>DE</b>	Germany	<b>LI</b>	Liechtenstein	<b>SD</b>	Sudan		
<b>DK</b>	Denmark	<b>LK</b>	Sri Lanka	<b>SE</b>	Sweden		
<b>EE</b>	Estonia	<b>LR</b>	Liberia	<b>SG</b>	Singapore		

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/NL 99/00638

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/12 A61K48/00 C12N5/10 A61K35/30 A61P25/28

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 14837 A (GENETIC THERAPY INC) 23 May 1996 (1996-05-23) page 4, paragraph 2 -page 10, paragraph 2 page 21, paragraph 3 -page 22, paragraph 1	1-5, 7
X	WO 97 16458 A (KOS HOLDINGS INC ;UNIV BOSTON (US)) 9 May 1997 (1997-05-09) page 3, line 33 -page 5, line 7 page 6, line 13 -page 10, line 35 page 14, line 19 -page 17, line 8 -/-	1-9

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- "A" document member of the same patent family

Date of the actual completion of the international search

15 May 2000

Date of mailing of the international search report

19/05/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Sitch, W

## INTERNATIONAL SEARCH REPORT

Int'l Application No

PCT/NL 99/00638

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	KAPLITT, MICHAEL ET AL: "Apolipoprotein E, A-beta- amyloid, and the molecular pathology of Alzheimer's disease: Therapeutic implications." RELKIN, N. R. 'EDITOR!; KHACHATURIAN, Z. 'EDITOR!; GANDY, S. 'EDITOR!. ANNALS OF THE NEW YORK ACADEMY OF SCIENCES, (1996) VOL. 802, PP. 42-49. ANNALS OF THE NEW YORK ACADEMY OF SCIENCES; APOLIPOPROTEIN E GENOTYPING IN ALZHEIMER'S DISEASE. PUBLISHER: N, XP002100573 * especially page 45, last paragraph * the whole document	1-9
X	DATABASE CHEMICAL ABSTRACTS 'Online! FILE SERVER STN KARLSRUHE ABSTRACT NO. 124:339812, FAZIO ET AL: "Murine bone marrow transplantation as a novel approach to studying the role of macrophages in lipoprotein metabolism and atherogenesis" XP002100575 abstract & TRENDS CARDIOVASC. MED., vol. 6, no. 2, 1996, pages 58-65,	1-3,5,7
A	BARGER ET AL: "MICROGLIAL ACTIVATION BY ALZHEIMER AMYLOID PRECURSOR PROTEIN AND MODULATION BY APOLIPOPROTEIN E" NATURE, vol. 388, 28 August 1997 (1997-08-28), pages 878-881, XP002093548 page 878 abstract	
A	EGLITIS ET AL: "HEMATOPOIETIC CELLS DIFFERENTIATE INTO BOTH MICROGLIA AND MACROGLIA IN THE BRAINS OF ADULT MICE" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, USA, vol. 94, 1997, pages 4080-4085, XP002100574 cited in the application page 4080 abstract	

# INTERNATIONAL SEARCH REPORT

International application No.

PCT/NL 99/ 00638

## Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claim 6 partially, insofar as it concerns an in vivo method, and claims 8 and 9 completely, are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/NL 99/00638

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9614837 A	23-05-1996	NONE	
WO 9716458 A	09-05-1997	AU 7552696 A	22-05-1997
		BR 9611329 A	28-12-1999
		CA 2236274 A	09-05-1997
		EP 0866799 A	30-09-1998
		JP 2000500332 T	18-01-2000